REMARKS

Claims 2-38 are pending, claim 1 having been canceled. No further amendments are proposed at this time.

The Office rejected claims 2-7, 9-23, 25-38 as allegedly obvious over Baugh et al. (Nucleic Acids Res., Vol. 29, No. 5e29 1-9, 2001; "Baugh") in view of Smith et al. (U.S. Pat. No. 6,027,945; "Smith"). According to the Office Action (at pages 5 and 7):

> "It would have been prima facie obvious to a person of ordinary skill in the art at the time the invention was made, to combine a method of producing aRNA as taught by Baugh et al. with a step of purifying the nucleic acid as taught by Smith et al. to achieve expected advantage of developing a sensitive and enhanced method of producing aRNA ... where the general conditions (suitable volume, incubation time) of a claim are disclosed in the prior art (Baugh and Smith et al), it is not inventive to discover the optimum or workable ranges by routine experimentation ... Applicants' arguments regarding Baugh et al. were found unpersuasive. The applicants' cited paragraphs disclosing 60 min incubation time is dependent on the concentration of starting RNA template, however, the same cited paragraph discloses less than 45 minutes incubation depending on the concentration of the starting template. Thus the incubation time varies based on the concentration used in the reaction."

Applicants respectfully disagree and traverse for the reasons below.

As acknowledged by the Office (Office Action at page 4), Baugh fails to disclose or suggest eluting nucleic acids from the solid phase in an elution volume of less than 50 microliters. Further, contrary to the Office's assertion and as set forth in applicants' previous reply, Baugh does not disclose a step of synthesizing a first strand cDNA or a second strand cDNA in a reaction that is completed in 45 minutes or less. The Office has not indicated precisely where a practitioner can find a description or suggestion of such a step in Baugh, but nevertheless appears to suggest that the reference discloses incubation times of less than 45 minutes. While Baugh does describe various individual incubation steps that individually takes less than 45 minutes, nowhere does it disclose or suggest synthesizing a first strand or second strand cDNA in 45 minutes or less, as recited in the instant claims.

In support of applicants' construction of the teachings of Baugh, attached hereto as Exhibit A is a copy of what applicants believe is a detailed description of Baugh's protocol,

which is available at the World Wide Web at mcb.harvard.edu/hunter (Exhibit A was printed by applicants' representative on July 16, 2008). With regard to Exhibit A, applicants point out that Baugh itself indicates (at page 2, left column, the end of the first full paragraph) that the webpage provided as Exhibit A contains a thorough description of the protocol used in the studies described in Baugh.

According to Baugh and Exhibit A, Baugh's method for producing a first strand cDNA or a second strand cDNA takes more than 45 minutes to complete. In fact, it takes at least 60 minutes to complete. For example, Baugh teaches synthesizing a first strand cDNA in a reverse transcription reaction that requires 60 minutes at 42°C (see Baugh, at page 2, right column, lines 1-2 of the first full paragraph; and Exhibit A, at page 6, about middle of the page). Baugh also discloses a step of producing a first strand cDNA involving different incubation times totaling 60 minutes, e.g., 40 minutes at 42°C, 10 minutes at 50°C and 10 minutes at 55°C; and 20 minutes at 37°C, 20 minutes at 42°C, 10 minutes at 50°C and 10 minutes at 55°C (see Baugh, at page 2, right column, lines 4-5 and lines 17-19 of the first full paragraph). In addition, Baugh describes synthesizing a first strand cDNA that takes at least 75 minutes to complete (20 minutes at 37°C, 20 minutes at 42°C, 10 minutes at 50°C, 10 minutes at 55°C, and 15 minutes at 65°C; see Exhibit A, at page 10, lines 1-7). Further, Baugh teaches a step of producing a second strand cDNA that takes at least 2 hours at 15°C or 14-16°C to complete (see Baugh, at page 2, left column, lines 13-14 of the second full paragraph; and Exhibit A, at page 10, line 19). Baugh does not teach or even suggest synthesizing a first strand cDNA or a second strand cDNA in a reaction that is completed in 45 minutes or less.

Smith fails to remedy the deficiencies of Baugh. Smith discloses a method of isolating biological target materials using silica magnetic solid particles (see Abstract). Smith does not teach producing a first strand cDNA or a second strand cDNA in a reaction that is completed in 45 minutes or less.

Smith also fails to disclose or suggest using an elution volume of less than 50 microliters. Smith discloses eluting nucleic acids with greater elution volumes, for example, 1 ml, 200 microliters, and 250 microliters (see column 15, lines 37-39, column 16, lines 56-57; and column

19, lines 33-35). Thus, Smith does not provide anything that would have led skilled practitioners to modify the method disclosed in Baugh in an attempt to arrive at applicants' method. Accordingly, Baugh and Smith, individually or in combination, fail to disclose or suggest every element of the instant claims, and therefore, fail to support a *prima facie* case of obviousness.

The Office Action (at page 5) seems to assert that a *prima facie* case of obviousness has been established, alleging that it would have been obvious for skilled practitioners to arrive at applicant's method by discovering the optimal work conditions of the methods described in Baugh and Smith via routine experimentation. Applicants respectfully disagree. Neither reference suggests eluting bound nucleic acids from solid phase material in a small volume, e.g., less than 50 microliters. As stated in the specification (e.g., at page 9, lines 11-23; and page 10, lines 14-30), such small elution volumes are advantageous for various reasons, e.g., efficiency, and obviating additional concentration steps. The Office has not pointed to anything to show that skilled practitioners would have been led to use small elution volumes without using applicants' own disclosure as a roadmap. Similarly, since neither Baugh nor Smith suggests modifying reaction times for synthesizing a first or a second strand cDNA, skilled practitioners would not have been led to reduce reaction times in the manner suggested by the Office. Further, as noted above, the shortest reaction time for synthesizing a cDNA strand disclosed in Baugh is 60 minutes, at least 25% more time than what is recited in the claims. As stated in the specification (e.g., at page 8, lines 3-9) and pointed out in applicants' previous reply, the claimed method reflects the surprising result that decreased reaction times are sufficient to allow amplification of RNA in the method. The Office has not pointed to anything in any of the cited references to show otherwise. Applicants submit that such a surprising result is sufficient to rebut any alleged *prima facie* case of obviousness, if indeed such a case could be established.

In view of the foregoing, claims 2-7, 9-23, 25-38 are not obvious over Baugh and Smith, individually or combined. Reconsideration and withdrawal of this rejection is respectfully requested.

The Office rejected claims 8 and 24 as allegedly obvious over Baugh and Smith, and further in view of Gerdes et al. (U.S. Pat. No. 6,872,527; "Gerdes"). According to the Office Action (at page 6), "... neither Baugh et al. nor Smith et al. teach random primers of nine nucleotides or longer. Gerdes et al. teach a method for genome wide amplification wherein the method utilizes 9-mer random primers to boost the amplification of entire or large fraction of the genome (see col. 26, line 23-64)." Applicants respectfully disagree and traverse.

The deficiencies of Baugh and Smith are as discussed above. Gerdes fails to rectify these deficiencies. The Office apparently cites Gerdes for disclosing genome wide amplification using 9-mer random primers (Office Action at page 6). Gerdes fails to teach producing a first strand cDNA or a second strand cDNA in a reaction that is completed in 45 minutes or less. It also does not teach eluting nucleic acids from the solid phase in an elution volume of less than 50 microliters. Accordingly, the Office has failed to establish a *prima facie* case of obviousness, since Baugh, Smith and Gerdes, individually and combined, fail to teach or suggest every element of the claims. Further, as pointed out above, applicants' method provides unexpected results sufficient to overcome any alleged *prima facie* case of obviousness. Therefore, applicants respectfully request that this rejection be reconsidered and withdrawn.

CONCLUSION

Applicants respectfully request that all claims be allowed. Applicants do not concede any positions of the Examiner that are not expressed above, nor do applicants concede that there are not other good reasons for patentability of the presented claims or other claims. No fees are believed to be due. However, please apply any other charges or credits to deposit account 06-1050, referencing Attorney's Docket No. 14255-052US1.

Respectfully submitted,

Date: July 17, 2008

Jeannie Wu, Ph.D.
Reg. No. 56,265

Fish & Richardson P.C.
225 Franklin Street
Boston, MA 02110
Telephone: (617) 542-5070
Facsimile: (877) 769-7945

21964530.doc

EXHIBIT A

aRNA Amplification

baugh@fas.harvard.edu

Please let me know about any improvements you make.

Contents

Introduction

This protocol is for generating amplified antisense RNA from limited quantities of total RNA. The protocol was originally developed by Eberwine and colleagues (Methods in Enzymology, vol. 303), and the development and analysis of this version are described by Baugh et al. (Nucleic Acids Research, in press). 5-20-fold mass conversions are typical for a single round of amplification starting with total RNA and yielding antisense RNA (aRNA, which is mRNA-derived). 200-400-fold amplification is typical for the second round starting with and yielding aRNA, allowing $>10^5$-fold amplification of mRNA.

The protocol is designed around maximizing yield and product length while minimizing template-independent side reactions. We found that template-independent reactions compete with the desired template-dependent reaction and that the consequences are more severe as less RNA template is used. Amplification products dominated by template-independent product result in greatly reduced sensitivity and compression of differences in microarray hybridization experiments. Most notably, the oligo-(dT) primer used in reverse transcription (RT) yields a high molecular weight product in the *in vitro* transcription (IVT) reaction independent of any cDNA template. We found this reaction to occur under all conditions tested and were unable to remove or destroy the primer without sacrificing the cDNA; the protocol is therefore designed around limiting the amount of primer used to start with. In addition, high molecular

weight, template-independent product is generated in the presence of biotinylated NTPs and the absence of any polymer when excessive amounts of T7 RNA polymerase activity are used. Template-dependent product of questionable molecular weight and limited functionality in downstream reactions can also be produced with excessive T7 RNA polymerase activity. Essentially, more yield is not always better.

The protocol limits the amount of primer used by employing small cDNA synthesis volumes. However, where there is a sufficient amount of material (at least 100 ng total RNA, depending on the application and the RNA), only a single round of amplification with routine reaction volumes is necessary. As a general rule of thumb, the 10 µl reverse transcription (RT) reaction is fine for 100 ng total RNA or more and the volume can be reduced by 1 µl per 10 ng less than 100 (10 ng total RNA per µl) down to 2 µl (1 µl reactions work, but they can be challenging). Because the point is to maintain a good ratio of RNA to primer, the concentration of primer used should be constant as reaction volumes are scaled down. The use of premixes in setting up the reactions is essential as they are scaled down. With a reliable p2 pipetor and some practice the small scale reactions are surprisingly reliable.

The random priming step in the second round of amplification severely limits the amount of 5' complexity represented in the resulting product. The consequences of this loss are dependent on the downstream application. For example, hybridizing to an oligonucleotide array (e.g., Affymetrix GeneChip®) or cDNA microarray that is not sufficiently 3' biased will result in a significant loss of sensitivity. To augment the loss of 5' complexity, we include T4 single strand nucleic acid binding protein (T4gp32) in all RT reactions. You may consider this unnecessary if you are doing only a single round of amplification or if you have the 3' most ends of transcripts represented on your array (e.g., cDNA macroarray). It is however better to include the T4gp32 since it enhances yield (as much as 50%) as well as processivity, and presumably makes the reactions more robust and therefore reproducible. In general, it is important that reverse transcription conditions are kept constant since differences in representation mat otherwise result. Even with the inclusion of T4gp32, it is recommended that you only make comparisons between samples (e.g., Cy3 vs. Cy5 in a single cDNA microarray hyb or an Affymetrix 2-file analysis) that have been amplified by roughly the same amount (at least the same number of rounds). If you must compare 1 round amplification products to 2 round products, T4gp32 will reduce the systematic differences between them resulting from their different molecular weight profiles. I have found different preps of T4gp32 to work better than others, so it is a good idea to get/prepare a single prep to complete a data set. T4gp32 is usually not commercially available at the high concentrations required, so there is a simple protocol included for concentrating it.

Successful amplification depends on a good RNA prep. You obviously want intact RNA, and it is critical that the RNA isolation procedure used be compatible with

downstream reactions. We found glycogen in particular to inhibit RT of larger templates (as if initiation and not processivity was affected) in a concentration dependent fashion. The inhibition was more severe than described by Life Technologies (Focus 16, number 4). Because the entire RNA prep is used and the cDNA synthesis volumes are small, what are ordinarily harmless amounts of glycogen severely inhibit the RT reaction. The RNA isolation procedure we have been having success with is described.

Because small amounts of nucleic acid are handled throughout the procedure it is imperative that Rnase-free technique be used. In addition, very sensitive methods are needed for quantification and characterization of RNA. A very sensitive fluorometry assay is therefore described and some suggestions on electrophoresis are offered.

RNA Isolation:

The described protocol is a derivation of Life Tech's TRIzol protocol, originally developed by Chomczynski and Sacchi (Analytical Biochemistry, 1987). A carrier is necessary for small amounts of starting material, and as described above, glycogen inhibits cDNA synthesis at high concentrations. Given the bizarre observations I have made regarding spurious polymerization products, I am weary of using nucleic acid carriers anywhere in the isolation/amplification procedure, so instead I use linear polyacrylamide (GenElute LPA from Sigma). I know people have also had success with Qiagen's Rneasy kit, though Qiagen admits that isolation efficiency drops off as very small amounts of material are used (a few thousand cells should be fine), and they recommend the use of a ribonucleic acid carrier. The protocol described suffers no practical lower limits and also results in a small, reproducible volume (5 µl, as opposed to the 30 µl volume the Qiagen protocol results in). The small, reproducible volume makes setting up small scale RT reactions less of a headache. Both protocols inactivate RNAses and are selective for RNA.

-Collect material into 30 µl or less of aqueous buffer (0.6 or 1.5 ml tube).
When using very small amounts of material it is important to include a negative control where there is no material added (e.g., dissection buffer minus cells) in addition to the true no template control which should be done with each set of amplifications.
-Either freeze the sample or go to the next step IMMEDIATELY.
-To fresh or frozen samples add 300 µl TRIzol reagent (Life Technologies) and mix thoroughly/homogenize by pipetting up and down several times.
I also like to shake the tubes in order to make sure I get any material that may have ended up other than in the bottom of the tube.
-Add water to approximately make up for however much less than 30 µl the sample started as.

-Add 5 µg linear polyacrylamide (GenElute LPA from Sigma).

When processing many samples, I make a premix of TRIzol plus LPA and water.

-Vortex 10 sec on high.

-Add 60 µl chloroform.

-Vortex 30 sec on high.

-Spin at full speed (~14K g) for 5 min.

-Transfer the aqueous phase to a clean (RNAse-free) 0.6 ml tube.

-Add 0.7-0.8 volumes of isopropanol (~180 µl).

-Mix well by shaking and vortexing.

-Precipitate overnight at -20°C.

-Spin at full speed (~14K g) for 20-30 min (I prefer 4°C) with the tube's hinge aligned with the rotor so that you can use it to find the pellet.

The pellet should be just barely visible as a transparent flake under the isopropanol after spinning.

-Carefully remove the supernatant by pipet.

-Wash once with 500 µl 75% ethanol.

The pellet should be visible (opaque) after the addition of ethanol.

-Spin at full speed for 5-10 min.

-Carefully remove the supernatant by pipet.

-Pulse spin (up to full speed) the tube to collect all residual ethanol at the bottom.

-Remove remaining supernatant by pipet.

-Allow the pellet to air dry for 1-2 min.

-Redissolve the pellet in 4-5 µl RNAse-free water.

I flick the side of the tube, vortex a few times, pulse the liquid back down and then let it sit at room temperature for ~5 min before putting the prep on ice or freezing it.

If you already know what volume RT reaction you will be using (and thus the amount of primer), go ahead and redissolve the pellet in water plus the appropriate mass of primer (e.g., redissolve the pellet in 5 µl of 4 ng/µl primer for a 2 µl RT reaction). If you want to quantify the yield of your RNA isolation and make decisions based on that, redissolve the pellet in 5 µl of water (no primer) and then use 0.5 µl in a fluorometry assay.

Amplification Notes

-No template control amplifications should always be included. Amplification products should be different from the no template controls in terms of both yield (yields should be at least 3-4 times the no template control) and electrophoretic mobility, or else they are probably not good.

-All cDNA synthesis enzymes can be purchased from Life Tech (separately or in a library synthesis kit ($$)). While I would only use SuperScript II reverse transcriptase (Life Tech), the source of other cDNA synthesis enzymes should not be too important. In particular, Epicentre also sells RNAse H and T4 DNA Polymerase at considerably lower prices, and they work just as well (be sure to use the same amount of activity since the concentrations may be different).

-The sequence of the (dT)-T7 primer is:

5'GCATTAGCGGCCGCGAAATTAATACGACTCACTATAGGGAGA$(T)_{21}$V 3'

(V = A, C, and G)

-The first part of the protocol is touchy and bears strongly on success. It is crucial that the volumes are as accurate as possible and that it is carried out as swiftly as possible.

-The volumes in round 1 are given twice. The first is for the 2 µl scale RT reactions and the one in parentheses is for the 10 µl scale. Some additional notes are given for the 2 µl scale.

Round 1 Amplification

-Combine 20 ng (100 ng) of the (dT)-T7 primer with your total RNA in a 0.6 ml tube (if you have not already at the end of the RNA isolation).

-Speed-vac the primer + RNA down to 1.0 µl (5.0 µl) WITHOUT DRYING COMPLETELY.

For the small scale you will have to monitor it very closely. I recommend both practicing and pipetting 0.5, 1.0 and 1.5 µl into separate tubes to have as a visual gauge of volume-I avoid measuring the volumes by pipetting for fear of losing material, though you can include some water controls and measure them directly. If you overshoot 1.0 µl, add water to bring the volume back up.

-Prepare RT premix.

RT Premix:

(per 5µl) 2.0 µl 5x 1st Strand Buffer
1.0 µl 100 mM DTT
0.5 µl 10 mM dNTP
0.5 µl T4gp32 (8.0 mg/ml) (USB)
0.5 µl Rnase Inhibitor (~20 U)
0.5 µl SuperScript II (100 U)
(?? µl dH2O to make up for evaporation during denaturation)

-Denature RNA + primer at 70°C for 4 minutes in a thermal cycler with a heated lid.

-Snap cool on ice, and keep on ice.

The volume may drop after denaturation. You should determine with controls beforehand how much it drops in your hands so as to add the appropriate amount of extra dH2O to the premix to make up for it.

- Add 1.0 µl (+ extra water) (5.0 µl) ice cold RT premix for a RT rxn volume of 2.0 µl (10.0 µl) and mix by pipetting.

-Incubate for 1 hr at 42°C in either a thermal cycler with a heated lid or an air incubator, but not in a water bath.

-Heat inactivate the rxn at 65°C for 15 min.

-Chill on ice.

-Prepare second strand synthesis (SSS) premix.

SSS Premix (round 1):

(per 65ul) 15 µl 5x Second-Strand Buffer (Life Tech, or homemade*)
1.5 µl 10 mM dNTP
20 U DNA Polymerase I
1 U *E. coli* Rnase H
5 U *E. coli* DNA Ligase
dH2O to 65 µl final volume

*5x Second Strand Synthesis Buffer:

100 mM Tris-HCl (pH 6.9)
23 mM $MgCl_2$
450 mM KCl
0.75 mM β-NAD
50 mM $(NH_4)_2SO_4$

-Add 13 µl (65 µl) ice-cold SSS premix for a 15 µl (75µl) SSS rxn volume, and mix by pipetting.

Be sure the premix is cold when you add it.

-Incubate at 14-16°C for 2 hours.

-Add 2 U (10 U) T4 DNA Polymerase, and mix by flicking and gentle vortexing.

-Incubate at 14-16°C for 15 min.

-Heat inactivate the reaction by heating it to 70°C for 10 min.

I go directly from 15°C to 70°C to avoid undesirable enzyme activities.

-Bring the volume up to 75 µl by adding 60 µl (0 µl) TE .

-Add 75 µl phenol:chloroform (1:1), and mix by pipetting vigorously.

-Transfer to prespun PLG Heavy, 0.5 ml tubes (Phase Lock Gel-Eppendorf), and spin 5 min at 13K rpm.

-Prepare BioGel P-6 MicroSpin Column (BioRad) per manufacturer's instructions.

That is, allow them to drain by gravity, and then spin them for 2 min at 1000 g.

-Transfer aqueous phase to prepared P-6 column and spin at 1000 g for 4 min recovering the flow through (~80 µl) in a clean 1.5 ml eppendorf tube.

Depending on what tube you want to set your IVT rxn up in, you may either transfer the flow-through to a new tube or leave it in the 1.5 ml tube. For instance, if you want to carry out the IVT rxn in a thermal cycler so as to have it held at 4°C rather than over-incubate.

-Add carrier (see below) and appropriate salt for precipitation (I have been using 3.5 µl (~1/25th vol) 5M NaCl) and mix by vortexing.

If you used the RNA isolation procedure described here, the LPA you added is still there and is sufficient at this step. If not, you should add either 5 µg linear polyacrylamide (GenElute LPA from Sigma) or 20 µg glycogen (Life Tech). If a second round of amplification is to be used (or other downstream RT rxns) then LPA is recommended over glycogen. LPA will slow microcon washes later (from about 12-14 min to 28-32 min for microcon-100s at 500 g and room temperature), but it does not inhibit RT.

-Add 2.5 vol's 95% ethanol (~220 µl) and mix well.

-Precipitate at -20°C for at least 2 hrs.

I let it go overnight.

-Spin at 13K rpm for 20 min.

-Carefully remove the supernatant.

-Wash 1x with 500 µl 70% ethanol, and spin for 5 min at 13K rpm.

-Carefully remove the supernatant.

-Pulse spin (up to full speed) the tube to collect all residual ethanol at the bottom.

-Remove remaining supernatant by pipet.

-Allow the pellet to air dry for 2-3 min.

-Prepare IVT premix.

IVT Premix: (prepare and hold at room temperature to avoid precipitate)
(per 40 µl)

16.5 µl DEPC ddH2O
4.0 µl 10 x Ampliscribe Buffer (Epicentre Ampliscribe Kit)
3.0 µl 100 mM ATP
3.0 µl 100 mM CTP
3.0 µl 100 mM GTP
3.0 µl 100 mM UTP
4.0 µl 100 mM DTT
1.5 µl Rnase Inhibitor (~60 U)
2.0 µl Promega HC T7 RNA Polymerase (80 U/µl)

For labeling, either include modified NTPs (with modification of the other NTP concentrations) or else just use one of the kits from Enzo Diagnostics according to their instructions. Read on for more discussion of IVT reagents.

The buffer and T7 RNA polymerase prep I use in my IVT rxns were changed as different artifacts became clear and different sources for each reagent were tested. The end result is a preferred IVT rxn that I know works very well but that is annoying in that it requires you to buy kits and not use all the components. Maybe one of the companies that sells these reagents will make their components available separately. The IVT premix I describe is the one I know works very well for straight amplification. For labeling I recommend a kit from Enzo Diagnostics. Alternatively, you can use the rxn I describe and include modified NTPs (from Enzo or elsewhere), with some adjustment in the NTP concentrations (somewhat lower to make labeled NTPs affordable). Combinations of reagents other than what I recommend may also work, but they should first be tested in a careful and controlled way, with an eye for the artifacts I have alluded to in the introduction. I can offer some experimentally determined conclusions:

T7 RNA polymerases: I know that Promega T7 RNA polymerase HC (80 U/µl) and the T7 RNA polymerase included in Enzo's Transcript Labeling Kits are both good for all IVT rxn's. I also know that Epicentre's very high concentration T7 RNA polymerase prep (2,500 U/µl) is NOT good for any IVT rxn-in spite of yielding so much mass. It may be the concentration of the enzyme (meaning it could be diluted and perform well) or it may be the prep itself-I gave up on it. Epicentre also sells a high yield IVT kit (Ampliscribe) that has a T7 polymerase prep in it at unknown concentration, which I am not sure about, but which stands a chance. I have good reason to believe Ambion's kit (MegaScript II) is not good.

IVT buffers: Epicenter's 10x Ampliscribe Buffer is what I use, except this buffer is only available in their Ampliscribe Kit. I also know that the 10x HY buffer included in Enzo's IVT kits is good, but these kits are sold for labeling rxns in particular, and so they include labeled NTPs you may not need. I do not know of a trustworthy buffer that is available other than in a kit. You might be able to make your own high yield buffer by

supplementing the standard 5x buffer that comes with most T7 RNA polymerases with inorganic pyrophosphatase (Sigma).

NTPs: I use either the NTPs that come with various kits (and have had no problems) or else the 100 mM NTPs sold by Promega.

-Add 20 µl (40µl) IVT premix.

If you started with micrograms of total RNA you may get a better yield if you use a 60 or 80 µl rxn volume.

-Resuspend the pellet in the premix by gently flicking and vortexing.

-Incubate at 42°c for 9 hr.

-Freeze or proceed.

-For purification by Microcon-100, add 480 µl TE (10 mM Tris, 1 mM EDTA, pH 8.0, DEPC-treated).

Microcons are slow with LPA in the sample, but they are what I have always used. Others have had success using Qiagen's Rneasy Columns (according to their RNA clean up protocol). I believe yield is equivalent. It is worth noting that carriers like LPA are washed away on Rneasy columns. In addition, something (a polysaccharide?) comes off microcon's with the sample at recovery that evidently causes streaking on gels.

-Transfer the 500 µl to a Microcon-100 and spin at 500 g down to <20ul (11-15 min at room temperature without LPA, 28-32 min with LPA).

-Add another 500 µl TE and spin again as before, and then repeat 1x (3 washes total).

Ending with a small volume in the final spin is ideal if you are going to go for a second round. In addition, I do the final wash with dH2O if I am going for a second round in order to keep the concentration of TE low.

-Measure/adjust volumes for downstream applications.

You may want to quantify the yield and analyze the products by electrophoresis, or you can just go to the next round.

Round 2 Amplification (if necessary)

-Add 0.5 µg random primers (mostly hexamers, Life Tech) to aRNA from round 1.

-Speed-vac aRNA + hexamers to 5.0 µl.

-Heat to 70°C for 5 minutes in a thermal cycler with a heated lid.

-Snap cool on ice.

-Sit at room temperature for 5 min.

-Prepare RT premix.

The same RT premix is used as in round 1.

-Add 5 µl room temperature RT premix, and mix by pipetting.

-Incubate by the following temperature protocol in a thermal cycler with a heated lid:

RT incubation (round 2):

20 min at 37c
20 min at 42c
10 min at 50c
10 min at 55c
15 min at 65c
hold at 37c

-Add 1 U Rnase H, and mix by vortexing gently.
-Incubate for 30 min at 37°C and then heat to 95°C for 2 min.
-Chill on ice and then spin briefly to collect condensation and return to ice.
-Add 100 ng (dT)-T7 (as 1 µl) while on ice.
-Incubate at 42°C for 10 min to anneal the primer.
-Prepare SSS premix (minus ligase).

SSS premix is identical to round 1 except MINUS LIGASE.

-Snap-cool samples on ice.
-Add 65 µl ice-cold SSS premix for a 75 µl SSS rxn volume.

Make sure both are cold before adding premix.

-Incubate at 14-16°c for 2 hr.
-Add 10 U T4 DNA Polymerase and mix by gentle flicking and vortexing.
-Incubate at 14-16°C for 15 min.
-Heat inactivate the reaction by heating it to 70°C for 10 min.

I go directly from 15°C to 70°C to avoid any undesirable enzyme activities.

-Carry out phenol:chloroform extraction, BioGel P-6 chromatography, and precipitation as in round 1.

See notes on carriers from round 1. I add 20 µg glycogen here rather than relying on the LPA to still be there, but I have no downstream RT rxns.

-Prepare IVT premix.

IVT premix (and considerations regarding substitutions) is identical to that described in round 1. Once again, for labeling, either include modified NTPs (with modification of the other NTP concentrations) or else just use one of the kits from Enzo Diagnostics according to their instructions.

-Add 40 µl IVT premix, and resuspend pellet as in round 1.
-Incubate at 42°C for 9 hr.
-Freeze or proceed to clean up and analysis.
-Wash 3x on Microcon-100 as after round 1.

Once again, Qiagen's Rneasy columns (RNA clean up protocol) are a safe alternative.

T4gp32 Concentration:

You can purchase T4gp32 from USB. You should talk to them and get the highest concentration lot # they currently have. In the past they have had lots at 4 and 6 mg/ml, which work well without concentration, but the optimal concentration is about 8-8.5 mg/ml. I have tried Ambion's T4gp32 and USB's *E. coli* single strand binding protein, but I did not get nearly as good results as with USB T4gp32 for either. You can use a larger volume than specified of a lower concentration prep in your RT reactions, but watch the glycerol concentration (up to 10% final glycerol is fine). There is nothing special about concentrating the T4gp32, you simply use a Microcon-10 (Millipore) per the manufacturer's instructions. Be sure to hold a few microliters out of the concentration to compare the optical density before and after concentration. Given the concentration of the prep you purchased, determine what fold concentration you need to reach the optimal concentration. Based on the volume loaded on the microcon you can determine what volume of flow-through will correspond to the optimal concentration. Pipet the desired flow-through volume of water into the flow-through recovery tube and mark the meniscus on the side of the tube. Remove the water, and use the mark to know when you are done concentrating. Alternatively, you can weigh the microcon with and without anything in it, determine the mass it should be with the desired amount of concentration, and then keep spinning until it reaches that mass. It has taken me from 1.5 hr to ~4 hr to get there (at 4°C). Recover the retentate by inverting the microcon in a new tube and spinning at maximum speed; you should also save the flow-through until you are done with the entire procedure. Precisely measure the volume of the retentate you recover by pipet (avoid bubbles). Measure the absorbance at 280 nm of the prep both before and after concentration (without a small volume cuvette (e.g., 50 or 100 µl) you will need a lot of your prep to do this); you should have no loss of mass in the concentration process.

You can assay your prep by running RT reactions with poly A+ RNA ladder (Life Tech) as template (I use 10 µl reactions with a couple hundred ng ladder as template), and running the ss cDNA products on either native or alkaline agarose gels. If not running an alkaline gel, you may want to hydrolyze the RNA in your sample before loading the gel so that you know you are looking at only cDNA. This can be done by adding NaOH to 100 mM and heating at 70°C for 10 min; it is probably a good idea to neutralize the sample with HCl before loading. In addition, you should see a shift in the bands probably resulting from the protein binding the products and slowing their mobility on native gels. On either gel, I do always see a very high band (between the top of a 1 kb DNA ladder and the well) and I do not know why. The presence of this band does not appear to depend on the RNA template used. The assay should be performed with a

range of T4gp32 concentrations so that you can ensure your prep is at the optimal concentration for adding the specified volume to the RT reaction (0.5 µl per 10 µl RT). You should get a good band with intensity similar to the others at 7.5 kb with very little interband smear. In exceptional preps you can get a good 9.5 kb band as well. You can dilute your prep with the flow-through you saved if need be. The prep can also be assayed using mRNA as template, though the differences in processivity are harder to see. With a good prep you should be able to see less low molecular weight product (a couple hundred bases, presumably representing 3' hard stops), and the top of the smear should extend noticeably higher on the gel.

Ribogreen Fluorometry Assay

Because measuring UV absorbance requires a relatively large amount of RNA, fluorometry is the preferred means of quantification before amplification and between rounds (50 µl quartz cuvettes with a 1 cm path (O.D. 0.05 = 100 ng RNA) are available for absorbance). The sensitivity of the assay depends on the instrument used as well as the assay volume. Hellma Cells sells a 12 µl quartz cuvette for fluorometry. The cuvette is 1 cm x 1 cm black quartz and fits in standard fluorometers. The dye RiboGreen is sold by Molecular Probes-it has a particularly high quantum efficiency when bound to RNA (excitation at 480 nm and emission at 520). On a good fluorometer I can get a signal to noise ratio of 2 with about 200-400 pg total RNA, and a trustworthy linear range between 0.5 and 20 ng using a 12 µl assay volume and ribogreen dye at 1:400.

Information on how to use the dye comes with it, and it is straightforward. You first want to dilute an RNA prep of known concentration and assemble a standard curve. The slope of this curve provides a conversion factor between measured signal and mass of RNA, except where the signal is very near background you should first subtract background. I make a good standard curve in the first place and do not remeasure it except when something about the set up changes (e.g., a new bulb in the fluorometer). I do always make sure the blank is the same, and when readings are high (or generally suspect) I dilute the sample 2-fold and measure. Because the dye also binds to DNA and fluoresces, you should make sure you are measuring what you think you are. You should also be on the lookout for other things that increase background fluorescence by doing negative controls and making the appropriate subtractions (e.g., nucleotides, Qiagen columns).

SYBR Gold Electrophoresis

The nucleic acid gel stain SYBR Gold (Molecular Probes) greatly enhances sensitivity of detection in agarose gels. I have found sensitivity to be best after staining in the recommended 1:10,000 dilution (1 x TBE or TAE) for at least 1 hour and then destaining briefly (~5-10 min, 1 x TBE or TAE). Sensitivity is also better in lower % gels (0.8% is fine), thinner gels, gels with narrower wells, and gels run shorter distances (digital imaging makes it easier). Taking care I can see and determine the molecular weight distribution of 20 ng of complex RNA or DNA (e.g., aRNA or cDNA), and I can see the structural bands of total RNA with as little as 10 ng. Be careful if using a ladder not to overload, or the signal will wash everything else out.

Gels can be used to check the integrity and to confirm the mass (as measured by fluorometry or absorbance) of total RNA, 1st round aRNA, and 2nd round aRNA at least initially if not whenever there is enough material. It is crucial when amplifying small amounts of RNA to compare real samples to no template controls by electrophoretic mobility as well as yield every time. Where digital imaging systems are available and densitometry can be easily performed, good gels with standards can at times replace fluorometry.